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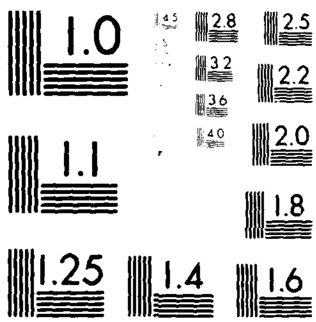

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Technical Report No. 19

Angiotensin I Converting Enzyme (Kininase II) of The Brush Border of Human and Swine Intestine

Prepared by

Ervin G. Erdös

For Publication In
Biochemical Pharmacology

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Mucosal brush border of human and swine small intestine is rich in angiotensin I converting enzyme or kininase II (ACE). The brush border of the intestinal mucosa was purified by centrifugation over a discontinuous glycerol gradient. Transmission electron micrographs showed that 90% of the isolated vesicles had a trilaminar membrane structure and glycocalix, characteristic to intestinal brush border. No significant contamination by other subcellular particles was evident. In the final purified preparation the brush border marker

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enzymes sucrase, trehalase, and alkaline phosphatase were enriched 23, 18 and 17 fold from human intestine and 27, 26 and 20 fold from swine tissue. ACE was highly concentrated in the human and swine brush border. The specific activity of ACE in the human and swine brush border fractions was enriched 17 and 7.6 fold over the crude homogenate. Kininase activity was demonstrated by bioassay. Captopril, the orally active specific inhibitor of ACE, inhibited the enzyme: its I_{50} was 3×10^{-9}M. Antibody to swine kidney ACE crossreacted with swine intestinal enzyme as shown in rocket immunoelectrophoresis indicating that the enzyme from kidney and intestine have common antigenic determinants and that the enzyme is concentrated on the brush border membrane. Because of the abundant presence of ACE in the intestine, interference in the functions of this enzyme may occur with chronic captopril therapy.

Angiotensin I converting enzyme (ACE; dipeptidyl carboxypeptidase; E.C. 3.4.15.1) cleaves dipeptides from the C-terminal end of peptides such as bradykinin, angiotensin I (1-4) and enkephalin (5). ACE is present in the plasma membrane of endothelial cells (2), and the epithelial brush border of renal proximal tubules is a rich source of ACE (6, 7). Because of morphological and biochemical similarities between renal tubular and intestinal brush borders (8, 9), we investigated the ACE content of human and hog intestinal brush border (10).+

These studies were also prompted by the use of the specific inhibitor of ACE, SQ 14225 or captopril, on a large scale in experimental animals and in hypertensive patients (11 - 14). Since this drug is given orally, it may inhibit ACE in the intestinal tract even before reaching the enzyme elsewhere in the organism.

ANGIOTENSIN I CONVERTING ENZYME (KININASE II) OF THE BRUSH BORDER OF HUMAN AND SWINE INTESTINE

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ABSTRACT

Mucosal brush border of human and swine small intestine is rich in angiotensin I converting enzyme or kininase II (ACE). The brush border of the intestinal mucosa was purified by centrifugation over a discontinuous glycerol gradient. Transmission electron micrographs showed that 90% of the ilsolated vesicles had a trilaminar membrane structure and glycocalix, characteristic to intestinal brush border. No significant contamination by other subcellular particles was evident. In the final purified preparation the brush border marker enzymes sucrase, trehalase, and alkaline phosphatase were enriched 23, 18 and 17 fold from human intestine and 27, 26 and 20 fold from swine tissue. ACE was highly concentrated in the human and swine brush border. The specific activity of ACE in the human and swine brush border fractions was enriched 17 and 7.6 fold over the crude homogenate. Kininase activity was demonstrated by bioassay. Captopril, the orally active specific inhibitor of ACE, inhibited the enzyme: its I_{50} was 3×10^{-9}M. Antibody to swine kidney ACE crossreacted with swine intestinal enzyme as shown in rocket immunoelectrophoresis indicating that the enzyme from kidney and intestine have common antigenic determinants and that the enzyme is concentrated on the brush border membrane. Because of the abundant presence of ACE in the intestine, interference in the functions of this enzyme may occur with chronic captopril therapy.

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These studies were also prompted by the use of the specific inhibitor of ACE, SQ 14225 or captopril, on a large scale in experimental animals and in hypertensive patients (11 - 14). Since this drug is given orally, it may inhibit ACE in the intestinal tract even before reaching the enzyme elsewhere in the organism.

EXPERIMENTAL

Materials

The chemicals used were obtained from the following sources. Hippurylglycylglycine was purchased from Vega Fox (Tucson, AZ, U.S.A.) and Bachem (Marina Del Rey, CA, U.S.A.). SQ 20881 (teprotide, Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and SQ 14225 (captopril, 2-thio-3-D-methyl-propanoyl-proline) were donated by Dr. Z. Horovitz of Squibb Institute (Princeton, NJ, U.S.A.). The sieve used for brush border preparations was from Tetko, Inc. (Houston, TX, U.S.A.). Crowle's double stain was obtained from Polysciences, Inc. (Warrington, PA. U.S.A.)

Tissues

Human small intestine was obtained within 3-6 h post-mortem. Swine small intestine was obtained from freshly slaughtered animals. Intestines were rinsed several times with 0.9% saline at 4^{o}, cut into one foot sections and frozen until used.

Brush border preparations

Human and swine intestinal microvillus vesicles were prepared according to the method of Schmitz et al. (15). In a typical preparation, approximately 10 grams of frozen mucosa was removed by gentle scraping with a glass slide and a 1% (w/v) homogenate was made in 50 mM-mannitol and 2 mM-Tris/HCl (pH 7.1). This and all subsequent procedures were performed at 4^{o}. The extract was homogenized in a Waring Blendor at full speed for 20 sec with a Powerstat variable transformer set at 90. After filtration through a 63 μm pore size mesh sieve, solid $CaCl_2$ was added slowly, with stirring, to a final concentration of 10 mM. After 10 min of gentle mixing, the homogenate was centrifuged at 2000 g for 10 min in a Sorvall RC2B refrigerated centrifuge. The resulting supernatant was recentrifuged at 20,000 g for 15 min to yield a small brownish pellet. This pellet was resuspended in 0.8 M-Tris HCl (pH 7.1) and slowly stirred for one hour. The mixture was then layered on top of a step gradient consisting of 37, 40, 42, 45 and 60% (v/v) glycerol in 50 mM-$MgCl_2$. After centrifugation at 63,000 g for 15 min in a Beckman SW 25.1 rotor of a preparative L5-65 ultracentrifuge, there were two bands visible in the gradient in addition to a pellet at the bottom. These bands and the pellet were collected individually, diluted with water and centrifuged in a Ti 60 rotor at 120,000 g for 90 min. The pellets were then resuspended in buffer and assayed separately.

Enzyme assays

ACE was assayed by incubating tissue fractions with 1 mM-hippurylglycylglycine in 100 mM-Tris/HCl (pH 7.4) containing 100 mM-NaCl at 37^{o} (1). ACE activity was calculated as the amount of substrate hydrolyzed that could be inhibited by 0.1 mM of the specific inhibitor SQ 20881. The amount of glycylglycine released was measured in a Beckman 121 amino acid analyzer. One unit of enzyme equals 1 nmole of substrate cleaved/min per mg protein. Kininase activity was determined by bioassay using the isolated rat uterus (6). In studies on inhibition, intestinal ACE was preincubated with captopril for 30 min before the addition of hippurylglycylglycine. The I_{50} was determined by plotting the log of inhibitor concentration against activity.

Brush border fractions were characterized by measuring marker enzymes. Alkaline phosphatase (E.C. 3.1.3.1.) was assayed according to the procedure of Linhardt and Walter (16). Sucrase (E.C. 3.2.1.26) and trehalase (E.C. 3.2. 1.28) were assayed according to the modification of Lloyd and Whelan (17) of Dahlqvist's method (18). Protein concentrations were determined by the method of Lowry et al. (19) using bovine serum albumin as a standard.

Transmission electron microscopy

Brush border vesicles of human small intestine were centrifuged to a pellet and fixed for two h in 2% (v/v) glutaraldehyde 100 mM potassium phosphate (pH 7.4) buffer. After post-fixation for two h in 2% (w/v) osmium tetroxide, the pellets were washed in distilled water, dehydrated through graded alcohols, infiltrated with propylene oxide and subsequently with Epon 812:propylene oxide (1:2) for storage overnight. Further infiltration and embedment of pellets in Epon 812 and polymerization at 60^{o} for 24 h rendered tissue capsules ready for sectioning with a diamond knife on a Sorvall MT-2 microtome. Sections were

mounted on bare copper grids, stained with uranyl acetate and lead citrate and examined with a Phillips electron microscope.

Rocket Immunoelectrophoresis

Immunoelectrophoresis was performed using 1.0 mm thick 1% (w/v) agarose gels on 1.5 x 83 x 102 mm glass plates. The electrode buffer was 37.5 mM-Tris 0.1 M glycine (pH 8.7) containing 0.3% NaN_3 and 1% (v/v) Triton X-100. The gel also contained 2% v/v monospecific swine kidney ACE antibody immunoglobulin. The monospecific antibody was obtained in rabbits using purified swine kidney ACE (20). Usually 10 µl of homogenate or brush border sample containing 3-20 µg of protein was applied to each well. Electrophoresis was carried out at 2 V/cm overnight. The gels were then pressed for 10 min (21) and rehydrated 0.9% in M NaCl for 20 min. Pressing and rehydration were repeated twice in NaCl and then the gels were rehydrated in water. The hydrated gel was stained for protein with Crowle's Double Stain. After one half hour in stain, the gels were destained with 0.3% acetic acid.

RESULTS

Marker enzymes

Although two bands and the pellet obtained in the glyerol gradient had marker enzymes and ACE, the membrane fraction isolated near the top of the glycerol gradient had the highest specific activity for brush border marker enzymes. In three experiments using swine intestine, the relative specific activity of sucrase, trehalase and alkaline phosphatase (compared to the specific activity of the original homogenate) was enriched twenty fold or higher (Table I). The corresponding enrichment of these enzymes in brush border isolated from human intestine was seventeen fold or higher (Table I). Recovery of brush border in the top layer, as estimated from recovery of marker enzymes, was 8 to 11% in both swine and human preparations.

Table I

Electron microscopy

One or two blocks were sectioned from each prep and 2-6 grids were examined from each of these blocks. These sample pellets contained both open and closed vesicles of varying sizes and densities (Fig. 1). Only about 10% of the vesicles were still filled with varying amounts of electron dense cytoplasmic material. Contaminating organelles such as nuclei, mitochondria, and lysosomes were rarely observed. The membrane vesicles were mainly ovoid in shape and had a trilaminar profile showing two dense layers of equal and uniform thickness. The granular layer or fuzzy coating on the vesicle membrane surface had the appearance of, and was suggested to be, the glycocalyx of the intact brush border membrane. Fig. 1.

ACE

The activity of ACE was concentrated in both the swine and human brush border preparations. The relative specific activity of the enzyme was 7.6 in swine brush border and 18 in the human preparations (Table1). Recovery of total activity ranged from 4 to 8%. Thus ACE is highly concentrated on the brush border of intestinal epithelial cells. The human intestinal brush border preparation also inactivated bradykinin as determined by bioassay. Bradykinin was inactivated at a rate of 7.7 ± 2.0 μg/min per mg protein and approximately 50% of this total kininase activity could be inhibited by captopril (10^{-6}M). Thus ACE on the human intestinal brush border, in addition to hydrolyzing hippurylglycylglycine, also inactivated bradykinin at a rate of 3.8 μg/min per mg.

Immunoelectrophoresis

To determine whether the swine intestinal ACE crossreacts with antibody to swine kidney ACE, brush border was solubilized with 1% Triton X-100 and was subjected to immunodiffusion against the ACE antibody. After crossreactivity was observed, varying amounts of swine intestinal homogenate and brush border

were subjected to immunoelectrophoresis against the antibody. In rocket immunoelectrophoresis (electroimmunoassay) the height of the rocket shaped precipitate is proportional to the amount of antigen (22). Increasing quantities of solubilized brush border (2, 4 and 8 μg) produced sequentially higher precipitation lines (1.4, 2.5 and 3.5 mm respectively). Alternately, only 9 μg of solubilized intestinal homogenate produced a visible precipitin line ($\leq$0.7 cm). Thus, this electrophoretic technique indicated both the presence and the enrichment of ACE in the intestinal brush border.

Inhibition

ACE on the isolated human brush border preparation was inhibited by captopril, the recently developed orally active inhibitor (11). The enzyme was inhibited 98% above 3×10^{-8}M concentration of the inhibitor, the I_{50} was 3×10^{-9}M.

DISCUSSION

These experiments have shown that the brush border of human and swine intestine is rich in ACE activity. The brush border preparations were purified according to an established procedure (15) and the identity and purity of the preparation was established by the enrichment of marker enzymes and by microscopic examination. The enrichment (17-27) fold and recovery (8-11%) of marker enzymes, which are localized almost entirely in the brush border, is similar to that reported by Schmitz, et al., (15). Since the enrichment and recovery of ACE was approximately half these values (8-18 fold and 4-8% respectively), ACE must be concentrated on but not exclusive on the brush border membrane. Thus the distribution of ACE is similar to that of

several other brush border peptidases which are present on both the brush border and within the cell (34). Since ACE enrichment and recovery was consistently higher in the human then in the swine preparations, ACE is probably more concentrated on human than on swine brush border. In addition, the absolute activity of ACE was 5 fold higher in human than in swine. Transmission electron microscopy of the isolated preparation showed the typical trilaminar membrane with attached "fuzzy coat" or glycocalyx. These are characteristic features of purified brush border vesicles (15) and brush border in situ (9, 23). In addition, no significant contamination by other subcellular organelles was apparent. Ninety percent of the vesicles were void of electron dense core material, and appeared empty. ACE is presumably bound to the membrane of the vesicles as it is with the isolated brush border of the kidney. Renal brush border maintains high ACE activity, even after the core material is removed from the microvilli (7).

The immunological cross-reactivity of swine intestinal ACE with swine kidney enzyme was shown by immunoelectrophoresis, since brush border intestinal ACE crossreacted with antibody to purified renal enzyme. The enzyme from these two tissues must have similar antigenic determinants.

ACE seems to be evenly distributed in the mucosa of various segments of the small intestine. In pilot studies we have not observed more than two-fold differences in the activity of samples originating from duodenum, jejunum or ileum. Others have found that crude intestinal homogenates of rabbit or rat contain high ACE activity (24, 25). After the completion of these experiments, we noted that Wigger and Stalcup (26), identified ACE in the intestinal epithelial cells of the rabbit embryo by immunofluorescence.

Inhibition of ACE by the orally active specific inhibitor captopril shows great promise in lowering elevated blood pressure (14, 27). Binding of the inhibitor by the intestinal ACE may affect its absorbtion and thereby its level elsewhere in the body. The human intestinal enzyme was inhibited by a very low concentration of captopril with an I_{50} of 3×10^{-9}M.

Chronic administration of the inhibitor may interfere with the functions of intestinal ACE. We can only speculate about the role of the enzyme in the intestine. Both kinins and angiotensin II are reported to have metabolic functions in the intestine. Kallikrein increases glucose absorption probably via the release of a kinin (28-30), while angiotensin II enhances fluid and sodium absorption from isolated intestinal sacs (31). Thus inhibition of intestinal ACE may affect these functions by increasing the concentration of bradykinin and decreasing that of angiotensin II.

Because ACE cleaves C-terminal dipeptides from a variety of substrates (2, 4), the intestinal enzyme may metabolize peptides other than angiotensin I or bradykinin. Proteolytic enzymes in the gastrointestinal tract can release peptide fragments of various length from proteins in the lumen. The actions of digestive enzymes such as pepsin, trypsin or chymotrypsin are determined mainly by the properties of amino acids adjacent to the peptide bond they cleave. Thus none of them would consistently release dipeptide substrates of dipeptidases present in high concentration in the intestine. ACE which cleaves the C-terminal dipeptides of polypeptides (2) may provide such dipeptides. Other enzymes such as dipeptidyl aminopeptidase (class 3.4.14.1), can also liberate dipeptides from the N-terminal end (32). Ubiquitous dipeptidases (E.C. Class 3.4, 13, 33), cleave dipeptides either on the cell surface or inside the cells to single amino acids which are absorbed from the intestine. Dipeptidases are present on the

membrane and in the cytosol of intestinal epithelial cells (34, 35). Some dipeptides may be absorbed through the cell wall even faster than single amino acids and it has been suggested that membrane hydrolases on the brush border may function as carriers (9, 36). Such a role for intestinal ACE in protein metabolism suggests that on the evolutionary scale, this function may antedate its action in regulating metabolism of vasoactive peptides.

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LEGENDS

Fig. 1.

Transmission electron micrograph of final pellet of purified human intestinal brush border showing closed intact vesicles, trilaminar membranes and attached glycocalyx.

TABLE I

ACTIVITY OF MARKER ENZYMES AND ANGIOTENSIN I

CONVERTING ENZYME IN ISOLATED INTESTINAL BRUSH BORDER

	Sucrase			Trehalase			Alkaline Phosphatase			ACE*		
	SA	RSA	R	SA	RSA	R	SA	RSA	R	SA	RSA	R
HOG												
Homogenate of Intestinal Mucosa	2.3 ± 0.4	1	100	5 ± 1	1	100	52 ± 10	1	100	2.5 ± 0.6	1	100
Isolated Brush Border	63 ± 20	27	11	130 ± 6	26	11	1030 ± 390	20	9	19.6 ± 5.8	7.6	4
MAN												
Homogenate of Intestinal Mucosa	7.1 ± 1.0	1	100	14 ± 5	1	100	79 ± 18	1	100	14.5 ± 8.2	1	100
Isolated Brush Border	162 ± 40	23	10	257 ± 127	18	8	1360 ± 160	17	8	260 ± 147	18	8

Specific activity (SA) is expressed as mean ± S.E.M. of three experiments. Relative specific activity (RSA) is (mean specific activity in the fraction)/(mean specific activity in the homogenate). Recovery of total activity (R) in the top layer of the gradient is given as percent. Activity = μmol/min per mg or * nmol/min per mg.

FOOTNOTES

*Established Investigator of the American Heart Association.

+Presented in part at the 1978 Fall Meeting of the American Heart Association in Dallas, Texas.

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